Oral treatments of *Echinococcus multilocularis*-infected mice with the anti-malarial drug mefloquine that potentially interacts with parasite ferritin and cystatin

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Abstract

In this study, we investigated the effects of oral treatments of *E. multilocularis* infected mice with the anti-malarial drug mefloquine, and identified proteins that bind to mefloquine in parasite extracts and human cells by affinity chromatography. In a pilot experiment, mefloquine treatment was applied 5 days per week and intensified by stepwise increasing the dosage from 12.5mg/kg to 200mg/kg during 4 weeks followed by treatments of 100mg/kg during the last 7 weeks. This resulted in a highly significant reduction of parasite weight in mefloquine-treated mice compared to mock-treated mice, but the reduction was significantly less efficacious as compared to the standard treatment regimen with albendazole. In a second experiment, mefloquine was orally applied in three different treatment groups at dosages of 25, 50 or 100mg/kg, but only twice a week, for a period of 12 weeks. Treatment at 100mg/kg had a profound impact on the parasite, similar to albendazole treatment at 200mg/kg/day (5 days/week for 12 weeks). No adverse side effects were noted. In order to identify proteins in *E. multilocularis* metacestodes that physically interact with mefloquine, we performed affinity chromatography of metacestode extracts on mefloquine coupled to epoxy-activated sepharose, followed by SDS-PAGE and in-gel digestion/LC-MS/MS. This resulted in the identification of *E. multilocularis* ferritin and cystatin as mefloquine-binding proteins. In contrast, when human cells were exposed to mefloquine-affinity chromatography, nicotinamide phosphoribosyl transferase was identified as a mefloquine-binding protein. This shows that mefloquine interacts with different proteins in parasites and human cells.

INTRODUCTION

The parasite *Echinococcus multilocularis* is an endoparasitic flatworm of the family Taeniidae. The life cycle of *E. multilocularis* is based on a predator-prey relationship. The definitive hosts are wild carnivores such as the red fox (*Vulpes vulpes*) and the arctic fox (*Alopex lagopus*), but the tapeworm also infects, and develops within the intestine of, domestic dogs and cats, increasing the infection pressure for humans [1, 2]. The definitive hosts shed eggs, which contain a first larval stage, the oncosphere. When taken up orally, oncospheres hatch as they reach the intestine, penetrate the intestinal wall and use the blood and lymphatic system for dissemination. They typically invade the liver, where they develop into the the metacestode stage, which causes human alveolar echinococcosis (AE). AE is distributed in the Northern hemisphere, the endemic areas stretching from Northern America through Central and Eastern Europe to Central and East Asia including Northern parts of Japan [1]. The increase in the urban fox populations in Central Europe, together with the high prevalence rate of *E. multilocularis* in foxes, has resulted in an increased environmental contamination with *Echinococcus* eggs, and as a consequence, this has led to an increased risk of transmission to humans [2].

Human AE manifests itself by tumor-like, infiltrative growth of metacestodes mainly in the liver, but other organs might also be affected. AE is often compared with a slow-growing liver cancer and if untreated the disease is usually lethal. The current strategy for treatment consists of surgical measures complemented by chemotherapy with mebendazole (MBZ) or albendazole (ABZ)). In inoperable cases, chemotherapy has proven to inhibit parasite proliferation acting parasitostatic, but benzimidazoles act rarely curative, resulting in life-long duration of treatment, high costs and elevated risk of side effects [3]. However, benzimidazoles have prolonged the average life expectancy of European patients at diagnosis from 3 to 20 years [3]. Nevertheless, alternative options for chemotherapy with parasiticidal activity are needed [4].

Mefloquine (MEF) is a synthetic analogue of quinine commonly used in the treatment and prophylaxis of chloroquine-resistant *Plasmodium falciparum* malaria [5, 6]. The mechanism of action of MEF against *Plasmodium* species has not been completely elucidated, but several investigations indicated a disturbance of haemoglobin metabolism, followed by the formation of an insoluble polymer, hemozoin, causing parasite death [5]. It is not known whether a hemozoin-related mode of action is relevant for the anti-echinococcal activity of mefloquine *in vitro*.

MEF also exhibited considerable efficacy against other helminths, such as *Schistosoma mansoni*, *S. japonicum*, *Opisthorchis viverrini*, *Brugia patei* and *B. malayi* [6-8]. We have earlier demonstrated the efficacy of MEF against AE in experimentally infected mice when the drug was applied intraperitoneally, but not when applied orally [9]. In this study we present two distinct oral MEF treatment protocols in *E. multilocularis* infected mice, and show that MEF treatment, when applied orally as a suspension in honey at a dosage of 100mg/kg twice a week, exhibits anti-echinococcal activity comparable to ABZ applied orally at 200mg/kg/day. We also show that (i) the iron-binding protein ferritin, and (ii) cystatin, a potential immune-modulator in parasite infections, bind to MEF, and thus possibly could be targeted by MEF in *E. multilocularis* metacestodes. In contrast, MEF-affinity chromatography of the human cancer cell line Caco2 results in binding of the enzyme nicotinamide phosphoribosyl transferase (NPT).

MATERIALS AND METHODS

Biochemicals and compounds. If not stated otherwise, all culture media and reagents were purchased from Gibco-BRL (Zürich, Switzerland) and biochemical reagents were from Sigma (St. Louis, Mo, USA). MEF was kindly supplied by Mepha Pharma AG (Aesch BL, Switzerland).

***In vitro* culture.** The culture of *E. multilocularis* (isolate H95) was carried out as previously described [10]. The human colon carcinoma cell line Caco2 was maintained as previously described by Müller et al [11].

Experimental infection of mice and treatments with MEF and ABZ. Female Balb/c mice (age 9 weeks; average body weight average of 25g) were housed in a temperature-controlled daylight/night cycle room with food and water *ad libitum*. Experiments were carried out according to the Swiss Federal Animal Welfare regulations (TschV, SR 455) under the licence number BE103/11. Metacestode solid fraction was obtained by extensive washing of *in vitro* cultured parasites with PBS, breaking the vesicles mechanically, followed by centrifugation [10]. The animals were randomly divided into experimental groups, and were then infected by intraperitoneal injection of 100µL of metacestode solid fraction in 100µL PBS.

The pilot experiment was performed with 15 mice, which were divided into 3 groups of 5 animals each. Compounds were formulated in 1% carboxymethylcellulose (CMC) at concentrations indicated below. Starting at 6 weeks post infection, mice were treated by gavage for a period of 11 weeks with either 100µL honey/CMC 1% (control group), 100µL ABZ (200mg/kg in honey/CMC 1%; ABZ group) or 100µL MEF at different concentrations (from 12.5mg/kg up to 200mg/kg in honey/CMC 1%; MEF group), basically 5 days per week and according to the treatment regimen depicted in Supplementary Figure 1. During the treatment, animals were carefully monitored and checked for clinical signs of impaired health such as weight loss, ruffled coat, hunched back, and changes in behavior (inactivity, nervousness), and the treatment regimen was adjusted accordingly. At the end of the treatment period of 11 weeks, the animals were euthanized, and after necropsy the parasite tissue was collected and the parasite weight measured. After pressing this material through a

metal tea strainer, 100µL of the resulting metacestode solution were re-injected into 2 mice per group to observe whether the parasite was still viable and regrowing.

Experiment 2 was performed with 39 mice of 9 weeks age, which were divided into 4 treatment groups of 8 animals each, and a control group of 7 animals. Starting at 6 weeks post-infection, mice were treated as follows: the control group received 100µL honey/CMC1%; the ABZ group received ABZ (200mg/kg) emulsified in honey/CMC1%; the MEF treatment groups were treated by gavage of MEF emulsified in honey/CMC 1% at 25mg/kg, 50mg/kg and 100mg/kg, respectively. The treatments were performed for a period of 12 weeks on 5 days/week for the ABZ-treated group and twice weekly for the MEF-treated groups. On those days where no MEF, but ABZ, was administered, mice from the MEF groups received honey/CMC1%. At the end of the study the animals were euthanized, and after necropsy the parasite tissue was collected and the parasite weight measured. The parasiticidal activity was assessed by *in vitro* cultivation of resected parasite-material.

The data was analyzed by use of the software R version 3.0.1. Upon removal of Grubbs outliers, data was analyzed by one-way ANOVA Bonferroni-adjusted *P* values calculated by Pairwise T-Test. Data was visualized by boxplot in Microsoft Office Excel 2010.

Coupling of MEF to epoxy-activated sepharose matrix. MEF was coupled to epoxy-activated sepharose matrix with a 12C spacer [11]. For this, 0.5g sepharose was extensively washed with distilled water and sedimented, followed by a two-step wash with coupling buffer (100mM $NaHCO_3$, pH 9.5). 20mg of MEF-HCl, solubilised in 1 ml DMSO, was added to 1ml of the washed epoxy-sepharose matrix and incubated for 72h on a a slow horizontal shaker at 37°C for 3 days in order to allow coupling to the epoxy-group. The resulting matrix was then transferred to a chromatography column (Novagen, Merck, Darmstadt, Germany) and washed with 20ml coupling buffer, followed by 1M ethanolamine pH 9.5 for 4h at room temperature in the absence of light, in order to block residual groups. Subsequently, the

column was extensively washed with PBS and PBS/DMSO (1:2) in order to remove unbound mefloquine. In additon to the MEF-sepharose column a mock-column was prepared containing epoxy-sepharose treated identically as described above but in the absence of MEF. The columns were stored in PBS containing 0.02% NaN_3 at 4°C.

Tandem-affinity chromatography of metacestode extracts and Caco2 cell lysates on epoxy-sepharose and MEF-epoxy-sepharose columns. Metacestode extracts were prepared from *in vitro* cultured parasites maintained under axenic conditions (i.e. in culture medium, but without rat hepatoma cells) and without FCS for 72h. After extensive washing with PBS, parasites were mechanically disrupted in PBS, centrifuged, and the pellet was resuspended in PBS containing Triton X-100 (0.1 %), 100µL Halt protease inhibitor (Thermo Fisher Scientific, Rockford, IL, USA) and 100 µL EDTA, and incubated at room temperature for 10 min. Following centrifugation at 4696 x g for 5min at room temperature. The supernatant (12ml) was collected and used for tandem-affinity chromatography. Cell free extracts from human Caco2 cells were prepared as described [11]. Extracts were first passed over the sepharose column without compound (mock column) at a flow rate of 0.2 ml/min, and the flow-through was collected, and then further loaded onto the MEF-sepharose matrix. Prior to elution, columns were washed with 8 column volumes of PBS. Elutions of both columns were done with PBS containing 10 mM MEF-HCl from a 100mM stock in DMSO. A second elution step was performed by applying 4ml of a low pH-buffer (100 mM glycine, pH 2.9). Fractions of 4ml volume each were collected and precipitated overnight with 80% acetone at -20°C. The precipitates were solubilized in 30µl Laemmli buffer and separated by 10% SDS-PAGE using a Hoefer minigel 250 apparatus (Amersham, GE Healthcare, Little Chalfont, United Kingdom). Proteins were visualized by silver staining [12]. For mass spectrometry (MS) analysis, colloidal Coomassie staining was applied overnight, and selected protein bands were cut out with a clean scalpel, placed in Eppendorf tubes containing ethanol/distilled

water (1:4) and stored at 4°C. The in-gel digestion/LC-MS/MS analysis was performed by the Mass Spectrometry and Proteomics Facility at the Department of Clinical Research of the University of Bern. The sequences obtained were blasted against standard protein databases for eukaryotes and against the database for *Echinococcus multilocularis* (http://www.genedb.org/Homepage/Emultilocularis).

Cytotoxicity assays. Toxicity of MEF on Caco2 cells was determined and IC_{50} values were calculated as previously described [11].

RESULTS

Oral application of MEF acts against *E. multilocularis* metacestodes in experimentally infected mice. Initial experiments carried out earlier had shown that MEF treatment of *E. multilocularis*-infected mice at25 mg/kg twice a week by oral route did not lead to a reduction in parasite weight, while intraperitoneal application had a profound effect [9].

In a pilot experiment, we applied an intensified MEF treatment protocol characterized by a stepwise increase of drug concentration (see Supplementary Figure 1). During an initial period of 9 days, 12.5mg/kg of MEF was applied 5 times, followed by 2 days without treatment and another 2 consecutive days of MEF treatment (see Supplementary Figure 1). No adverse effects were noted, thus the dose was increased to 50mg/kg for another 3, 5 and 4 days treatments, all within a 16 days period (see Supplementary Figure 1). Again, no adverse effects or behavioural changes were noted. The dose was increased to 100mg/kg for 2 days, and then increased to 200mg/kg for another 2 days, which then resulted in rapid weight loss (see Supplementary Figure 1) and ruffled coats in three of five mice, and one mouse died on day 30. Subsequently the MEF dosage was kept at 100mg/kg, and was applied as such 31

times during the residual 48 days, during which the mice recovered body weight again and did not show further side effects. No changes in body weight or any other adverse effects were noted in ABZ treated mice. At the end of the 11 week experimental treatment, mice had ingested a total of 4.0875g/kg of MEF per mouse or 10.8g/kg of ABZ (ratio MEF : ABZ = 1:2.45), respectively. Measurement of parasite weights at the end of the treatment period of 11 weeks showed there was a significantly beneficial effect of MEF treatment compared to the non-treated control group (p=0.0016, Fig. 1A). However, the intensified MEF treatment was not as efficacious as daily ABZ treatment, as the parasite weights in the MEF treatment group were significantly higher compared to the ABZ treatment group (p = 0.0495) (Fig. 1A). ABZ treatment led to a highly significant reduction of cyst weight (p=7.4E-05). Drug treatments did not act parasiticidal, since reinfection with cyst material of two mice per group resulted in parasite-regrowth in all cases (data not shown).

In experiment 2, MEF was applied to mice orally by feeding of a MEF-honey suspension, at drug concentrations of 25 mg/kg, 50mg/kg or 100mg/kg only twice a week. As reference control, mice were treated with ABZ (200mg/kg on 5 days per week). No adverse side effects, no weight loss and no behavioural changes could be detected in any of the animals during the time of drug treatment. During these 12 weeks, mice treated with 100mg/kg of MEF twice per week ingested a total of 2.4g/kg of MEF, and mice treated with ABZ were given a total of 12 g/kg of ABZ (MEF : ABZ = 1:5). Measurement of the parasite burden at the end of the treatment period of 12 weeks showed that oral MEF treatment at 100mg/kg twice a week was as efficacious as daily ABZ treatment (200mg/kg/day) (Fig. 1B). The reduction in parasite burden was a significant in the ABZ-treated group (p=0.018) and in the MEF (100mg/kg) treated mice (p=0.018), while MEF treatment at lower doses (25 and 50mg/kg) showed a non-significant reduction. None of these drug treatments acted parasiticidal, since *in vitro* culture of parasite material from all the treatment groups resulted in regrowth of metacestode vesicles.

Epoxy-sepharose-immoblilized MEF binds *E. multilocularis* ferritin and cystatin. In order to identify proteins that interact with MEF, *E. multilocularis* metacestode fractions were exposed to sepharose-bound MEF. The fractions eluted from the mock-sepharose and the MEF-sepharose columns were analyzed by SDS-PAGE and silver staining (Fig. 2). After loading *E. multilocularis* extract onto the mock-sepharose and subsequent MEF affinity chromatography of the flow-through, the columns were washed with MEF-containing elution buffer and subsequently with low pH buffer. As seen in Fig. 2, washing of the mock-sepharose column with MEF-containing elution buffer did not result in removal of any proteins. The elution with low pH-buffer revealed that considerable amounts of *E. multilocularis* proteins bound to the sepharose matrix and were released again at low pH. MEF-sepharose affinity chromatography of the mock-sepharose-flow-through fraction yielded a major double-protein band of around 20kDa eluting with MEF-containing buffer. Subsequent washing with low-pH buffer did not result in further release of proteins (Fig. 2). The two bands of the MEF elution (indicated with arrows as 1 and 2, Fig. 2) were cut out and analysed by in-gel digestion/LC-MS/MS. Both protein bands were identified as ferritin and cystatin from *E. multilocularis* (Table 1; EmuJ_000382200.1 and EmuJ_000849600.1).

Epoxy-sepharose-immoblilized MEF binds to human nicotinamide-phosphoribosyl-transferase (NAPRT)

Human cancer cells have been shown to be highly susceptible to antimalarials including MEF [13]. The susceptibility of the human colon cancer cell line Caco2 against MEF was assessed *in vitro*. The IC_{50} for non-confluent Caco2 cells was 0.7±0.1µM, and confluent cells exhibited an IC_{50} of 3.6±0.7µM. MEF binding proteins in human cells were identified using the identical approach as for *E. multilocularis* metacestodes, resulting in the elution of a single major band of approximately 50kDa. Subsequent elutions with with low-pH buffer were

devoid of major bands (Fig. 3). The 50kDa band was subjected to analysis by mass spectrometry and identified as the 491 amino acid protein nicotinamide-phosphoribosyl-transferase (NAPRT; P43490).

DISCUSSION

As the pilot study showed, a stepwise increase of oral MEF input was significantly effective, but had detrimental effects, resulting in a lower efficacy of MEF compared to ABZ, and obvious adverse side effects once higher concentrations than 100mg/kg were applied.

The second study demonstrates that oral application of mefloquine at 100mg/kg twice a week for 12 weeks in experimentally infected mice acts against *E. multilocularis* metacestodes in a similar manner as oral application of albendazole (200mg/kg/ on 5 days per week). Mefloquine applied at 25mg/kg or 50mg/kg was ineffective, confirming results from earlier studies [9]. At the lower concentrations the bioavailability of MEF was probably too low to elicit a therapeutically active serum level, while an increased MEF input resulted in serum levels that induced toxicity. For comparison, high single oral MEF doses of 200 to 400mg/kg were necessary to achieve significant worm burden reductions in *S. mansoni*- and *S. japonicum*-infected mice. However, for extended treatments (as they would be anticipated in AE patients), the application of MEF could be problematic. Daily doses above 12.5mg/kg have been reported to cause epilepsy or impairment of motor performance, and increased lethality was observed in mice at dosages of 30mg/kg and more [14-18]. Despite of the reported adverse effects of MEF when applied in high doses [18], no adverse effects were observed in the behavior or overall health of the animals throughout the treatment phase in experiment 2, most likely due to the fact that treatments were restricted to twice a week. In any case, this study shows the critical role of dosage to achieve anti-parasitic activity without causing adverse effects. Further it has to be highlighted that unlike shown in a previous *in*

vitro study [9], *in vivo* MEF treatment did not kill the parasite completely. Full parasiticidal activity, and especially toxic activity against *E. multilocularis* stem cells, would be necessary to completely kill the parasite and avoid the necessity of life-long drug uptake [19].

Research on novel compounds for the treatment of secondary AE in mice should focus on drugs or drug classes that are already marketed and/or in clinical development, and these are preferably cancer drugs or broad-spectrum anti-parasitic compounds. In this respect, MEF is commercially available, FDA-approved, and has been extensively characterized in terms of bioavailability, pharmacokinetics, and toxicity. However, the adverse effects reported upon the uptake of MEF are a problematic issue. The drug is known to cause neurotoxicity, and neuropsychiatric effects have been reported. Thus, it would be important to further investigate derivatives of MEF, and identify those compounds with similar anti-parasitic activity but with an impaired ability to pass through the blood-brain barrier. In addition, it has been shown that MEF is metabolized only slowly, with a plasma half-life in humans of 2-4 weeks, and metabolization occurs in the liver. During long-term treatments, as they would be anticipated in human patients suffering from AE, which represents mainly a liver-associated disease, this could cause significant adverse effects.

The interaction of *E. multilocularis* ferritin and cystatin with MEF by affinity chromatography could indicate a possible anti-echinococcal mechanism of action for the drug. Both proteins were detected in two bands that migrated closely together on SDS-PAGE. Ferritin is an important intracellular iron-storage protein, generally composed of 24 subunits of around 20kDa and these 24 subunits are made up by ferritin heavy and light chains [20]. In *E. multilocularis* there has only one gene been identified for ferritin. Invertebrate ferritins show greater similarity towards the vertebrate heavy chains than towards the light chains, and ferritin heavy and light chains have been identified in nematodes and trematodes [21]. Thus, possibly the light chain homologue has not been identified in the *E. multilocularis* genome so

far due to more pronounced sequence divergence. Further, *E. multilocularis* ferritin has a predicted size of 19.8kDa that is in accordance with the apparent molecular weight of the protein bands on the silver stained gel. Ferritin is responsible for the maintenance and transport of iron in a non-toxic form In addition, ferritin heavy chain possesses ferroxidase activity, converting Fe^{2+} to Fe^{3+}. Fe^{2+} can interact with hydrogen peroxide, resulting in highly damaging hydroxyl radical formation [20]. Thus, we propose that there could be a link between iron metabolism of the parasite and the mode of action of MEF in *E. multilocularis* metacestodes. *Plasmodium* species use haemoglobin, an extracellular Fe-containing protein of vertebrates, as a nutrient source. Interestingly, the mode of action of MEF in plasmodia is linked to binding of the heme part of haemoglobin [5], which implies that the Fe-containing residue is crucial both in plasmodia and *Echinococcus* for the action of MEF.

Cystatins are inhibitors of cysteine proteases of the C1 family. However, in the field of parasitology, cystatins are given special attention, because they regulate the activity of proteases involved in parasite migration, and they are immunomodulators of parasite infections [22]. Interestingly, in the trematode *Schistosoma mansoni* cystatin is involved in the regulation of haemoglobin degradation [23]. The *E. multilocularis* cystatin has a predicted molecular weight of 30.9kDa. This is not in accordance with the apparent molecular weight of the protein bands on gel of about 20kDa, thus the detected band could have resulted from protein degradation.

One has to keep in mind, however, that MEF may bind to proteins that do not contain iron. In *Schistosoma*, enolase has been identified as a MEF binding protein [24]. Here, we show that human NAPRT binds to MEF. NAPRT, also known as visfatin, is a key enzyme in the NAD salvage pathway and acts as an immunoregulatory cytokine [25]. By activating sirtuin, NAPRT promotes cell longevity [26] and has neuroprotective effects [27]. Conversely, inhibition of NAPRT induces apoptosis rendering this protein an interesting target for the development of anti-tumor agents [28].

MEF is not the only anti-malarial exhibiting anti-echinococcal activity. For artemisinin derivatives such as antimalarial peroxides it has been shown that peroxide-bond reduction by hemoglobin-derived Fe^{2+} produces short-lived alkoxy radicals, which quickly rearrange to a carbon-centred radical that alkylates heme, forming heme-drug adducts [29]. Similar to mefloquine, these artemisinins also have a high activity against schistosomes, which like plasmodia, digest haemoglobin, in this instance by ingestion of erythrocytes [30]. The anti-schistosomal properties of MEF have also been established, and the presence of haemin was shown to enhance the treatment efficacy [6, 30]. However, MEF-affinity chromatography of *Schistosoma mansoni* schistosomula had revealed that the glycolytic enzyme enolase binds to the drug [24], and this study had demonstrated a potential role of MEF as an inhibitor of glycolysis in stages where heme degradation was not relevant.

In conclusion, MEF is effective in the treatment of AE when administered orally at high doses, but only twice a week. Moreover, the drug presents the advantages of being commercially available and currently used for the prophylaxis and treatment of malaria. Despite much discussion, the mechanism of action of MEF is not yet clarified for *Plasmodium* species. For *E. multilocularis*, we have identified ferritin and cystatin as two MEF-binding proteins, which are clearly different from NAPRT pulled out from human cell extracts, and their role as drug targets for MEF in these parasites will be further evaluated in future studies.

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Figure legends

Figure 1. Effects of oral treatments of *E. multilocularis* infected mice in terms of recovered parasite weight. In the pilot experiment (A), oral application of albendazole (ABZ) was compared with mefloquine treatment (MEF) for a period of 11 weeks at dosages as indicated in Supplementary Fig. 1. The control group received oral treatment with solvent only (honey/CMC 1%). Note that the differences in parasite weights between the ABZ, MEF and control group are all significantly different from each other. In experiment 2 (B) mice were treated for a period of 12 weeks with either honey/CMC 1% (control), albendazole (ABZ; 200 mg/kg daily on 5 days per week) or mefloquine (MEF; 25, 50 or 100 mg/kg twice per week). Note the significantly reduced parasite weight in the ABZ and MEF (100mg/kg) group, while MEF was ineffective at 25 and 50 mg/kg. For statistical analysis, Grubbs outliers were removed, and further testing performed with ANOVA and Bonferroni-adjusted *P* values calculated by Pairwise T-Test.

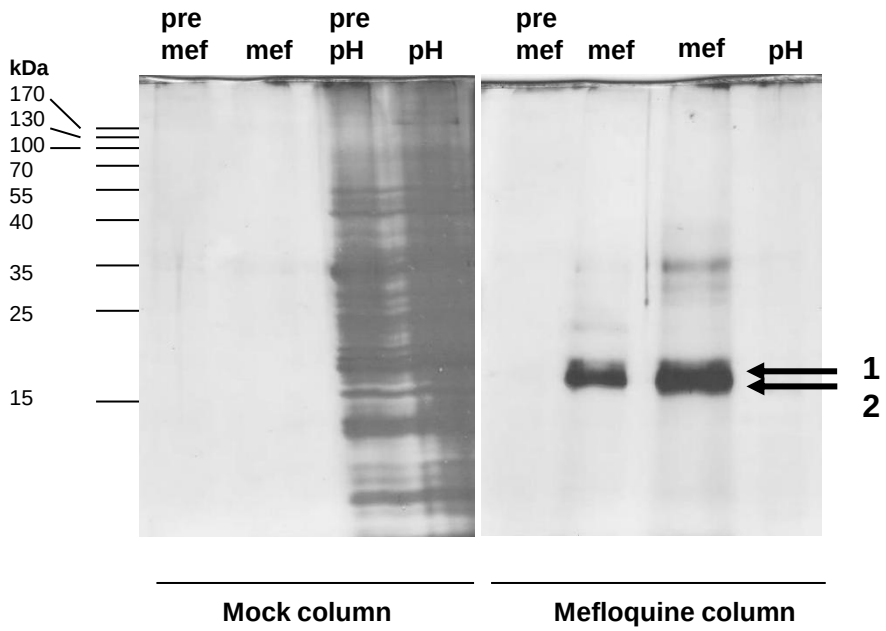
Figure 2. SDS-PAGE visualization of proteins from soluble *E. multilocularis* metacestode extract eluted after affinity chromatography with mefloquine bound to epoxy-activated sepharose. Silver stained gel showing fractions eluted from the unloaded epoxy-sepharose column (Mock column) and the subsequent mefloquine column. Pre-mef = flow-through fraction obtained prior to mefloquine elution; mef = fraction eluted with excess mefloquine; pre-pH = fraction obtained prior to pH-shift elution; pH = fractions eluted with low-pH buffer. The two bands eluted with excess mefloquine (indicated by arrows 1 and 2) were cut out and subjected to LC-MS/MS analysis.

Figure 3. SDS-PAGE visualization of proteins from soluble human colon cancer (Caco2) cell extract eluted after affinity chromatography with mefloquine bound to epoxy-activated sepharose. Silver stained gel showing cell extract (CE) loaded onto the columns, flow through (FT) and wash fraction (pre-mef = flow-through fraction obtained prior to mefloquine elution. mef = fraction eluted with excess mefloquine; pH1 and pH2 = fractions eluted with low-pH buffer. The band eluted with excess mefloquine (indicated by arrow) was cut out and subjected to LC-MS/MS analysis.

Supplementary Figure 1. For the pilot experiment, the treatment regimen (A) and the development of mean body weights measured during treatments (B) are presented. Albendazole (ABZ) dosage was kept continuously at 200 mg/kg per application, while the dosages for mefloquine (MEF) varied. Monitoring of the mean body weight during the treatments shows a marginal impact of ABZ, while for MEF the high dosage of 200 mg/kg twice resulted in weight loss and subsequent death of one animal on day 30 (arrow). The remaining mice recovered when MEF-treatment was continued at lower dosage (31 x 100 mg/kg during the next 48 days) and these mice finally achieved a similar body weight as ABZ-treated mice.

AC	Description	PMSS band 1	PMSS band 2	Protein score	Cov %	#PSMs	#Peptides
EmuJ_000382200.1	ferritin	3875.722	3655.734	1758.516	93.68	1111	81
EmuJ_000849600.1	proteinase inhibitor I25, cystatin	31.046	5.346	1367.22	77.09	386	64
EmuJ_000684200.1	Lipid transport protein, N terminal		5.922	60.982	2.21	7	7
EmuJ_000720500.1	ATP synthase subunit alpha, mitochondrial		5.269	17.903	4.2	2	2

Table 1. Analysis of in-gel digestion LC-MS/MS of two bands (1 and 2), the two mefloquine-binding proteins eluted by affinity chromatography using mefloquine-epoxy-sepharose. Represented are the accession numbers (AC) of the identified proteins in the database http://www.genedb.org/Homepage/Emultilocularis; their description; the PMSS values that are the sum of z-scores of all peptides of a protein, thus representing a semiquantitative measure of the abundance of the respective proteins; the protein score; the percentage of coverage (cov %) of each protein; the number peptide scan matches (PSMs); the number of peptides.



Graphical abstract

Figure

Figure 1

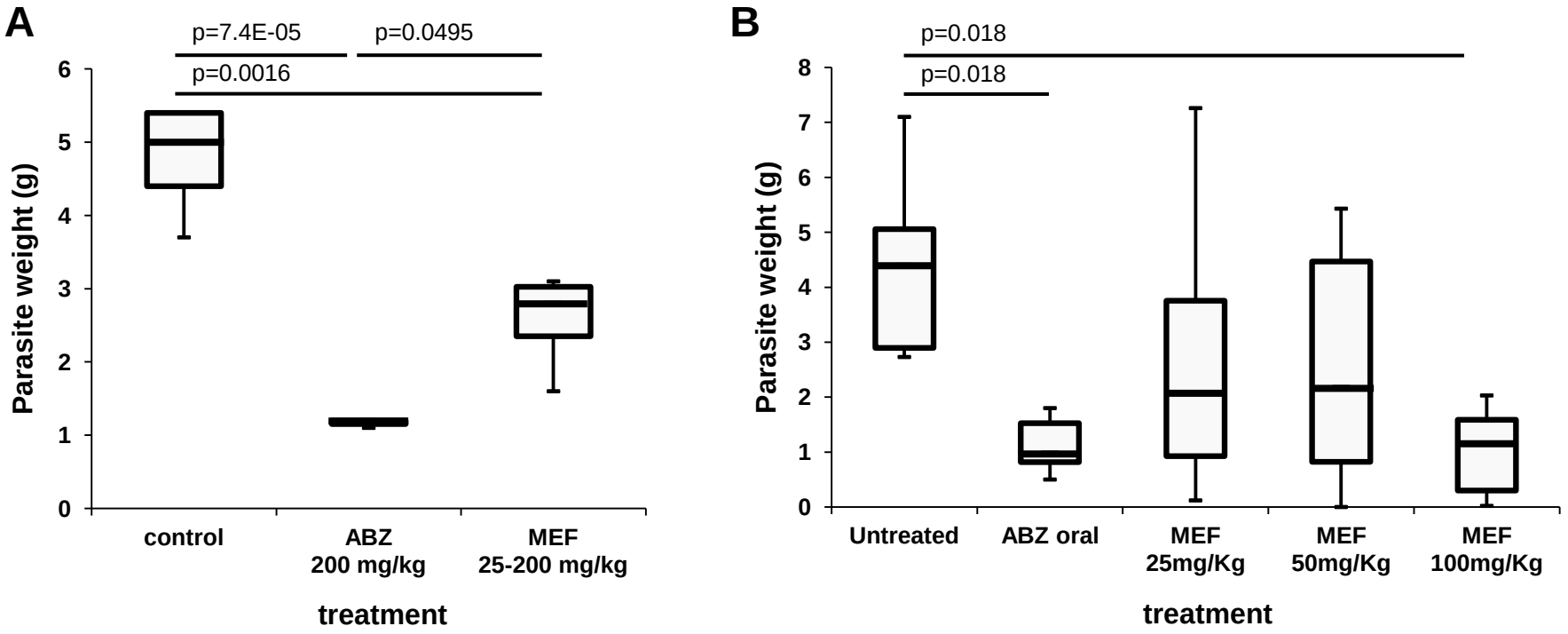
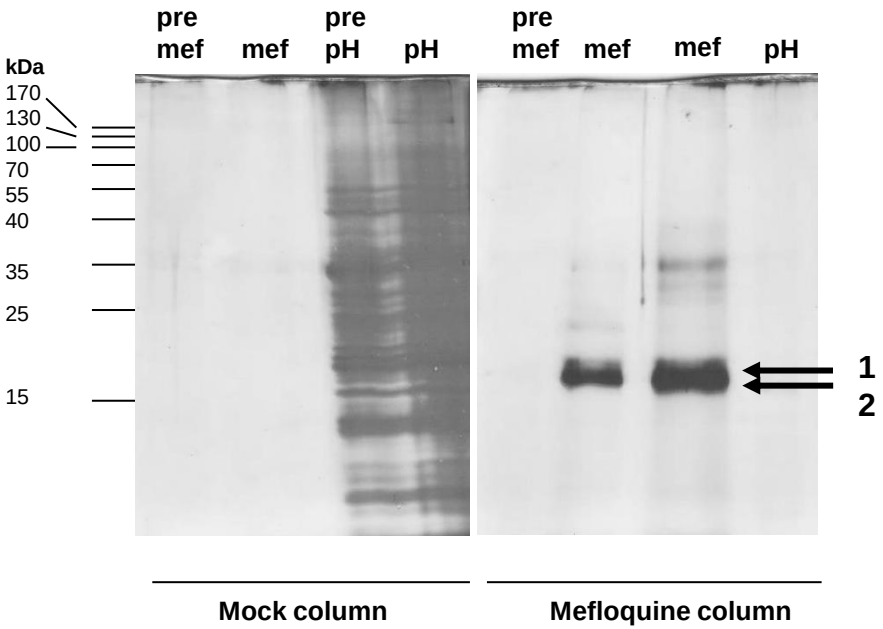


Figure 2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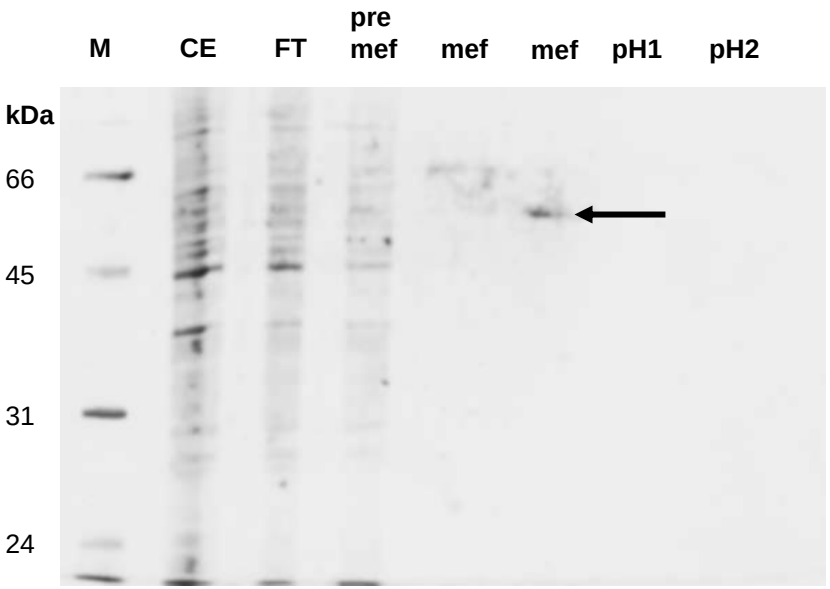
35
25
15
1
2
Mock column
Mefloquine column

Figure 3



Supplementary Figure 1